

Immunotherapy with MVA-BN[®]-HER2 induces HER-2-specific Th1 immunity and alters the intratumoral balance of effector and regulatory T cells

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Abstract MVA-BN[®]-HER2 is a new candidate immunotherapy designed for the treatment of HER-2-positive breast cancer. Here, we demonstrate that a single treatment with MVA-BN[®]-HER2 exerts potent anti-tumor efficacy in a murine model of experimental pulmonary metastasis. This anti-tumor efficacy occurred despite a strong tumor-mediated immunosuppressive environment characterized by a high frequency of regulatory T cells (T_{reg}) in the lungs of tumor-bearing mice. Immunogenicity studies showed that treatment with MVA-BN[®]-HER2 induced strongly Th1-dominated HER-2-specific antibody and T-cell responses. MVA-BN[®]-HER2-induced anti-tumor activity was characterized by an increased infiltration of lungs with highly activated, HER-2-specific, CD8⁺CD11c⁺ T cells accompanied by a decrease in the frequency of T_{reg} cells in the lung, resulting in a significantly increased ratio of effector T cells to T_{reg} cells. In contrast, administration of HER2 protein formulated in Complete Freund's Adjuvant (CFA) induced a strongly Th2-biased immune response to HER-2. However, this did not lead to significant infiltration

of the tumor-bearing lungs by CD8⁺ T cells or the decrease in the frequency of T_{reg} cells nor did it result in anti-tumor efficacy. In vivo depletion of CD8⁺ cells confirmed that CD8 T cells were required for the anti-tumor activity of MVA-BN[®]-HER2. Furthermore, depletion of CD4⁺ or CD25⁺ cells demonstrated that tumor-induced T_{reg} cells promoted tumor growth and that CD4 effector cells also contribute to MVA-BN[®]-HER2-mediated anti-tumor efficacy. Taken together, our data demonstrate that treatment with MVA-BN[®]-HER2 controls tumor growth through mechanisms including the induction of Th1-biased HER-2-specific immune responses and the control of tumor-mediated immunosuppression.

Keywords Immunotherapy · Modified Vaccinia Ankara (MVA) · Th1 · Immunosuppression · Breast cancer · Regulatory T cells · HER-2

Introduction

MVA-BN[®]-HER2 is currently being developed as a candidate for breast cancer immunotherapy and has shown biological activity in ongoing Phase I clinical trials (NCT0048277). MVA-BN[®]-HER2 is a Modified Vaccinia Ankara-based recombinant vaccine vector derived from MVA-BN[®] (IMVAMUNE[®]), a highly attenuated clonal virus currently in development as a smallpox vaccine [1]. MVA-BN[®]-HER2 encodes a modified form of human epidermal growth factor receptor 2 (HER-2), referred to as HER2. HER2 comprises of the extracellular domains of HER-2 and was further modified to encode two promiscuous T helper cell epitopes from tetanus toxin. This modification has been shown to improve its immunogenicity in a tolerant environment [2].

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Successful cancer immunotherapy will most likely require the induction of comprehensive innate and Th1 adaptive immune responses that provide effector mechanisms able to directly kill tumor cells while combating the inflammatory/immunosuppressive responses induced by the growing tumor. In this regard, MVA-BN[®] has been shown to confer protection in animal models of smallpox through the induction of strong Th1 adaptive as well as innate immune responses [3–7]. These intrinsic immune properties are beneficial features to mediate adequate immune responses to MVA-BN[®]-encoded transgenes expressing tumor targets. This was demonstrated herein, by comparing the induction of HER-2-specific immune responses mediated by either MVA-BN[®]-HER2 or HER2 formulated in Freund's adjuvant. When tested in a murine model of experimental pulmonary metastasis (CT26-HER-2), MVA-BN[®]-HER2-mediated immune responses translated into potent anti-tumor efficacy, while HER2 formulated in Freund's adjuvant had only modest effects.

Multiple cell types can exert immunosuppressive functions within tumors, thereby hampering the development of successful immunotherapies [8–12]. Among these, regulatory T cells (T_{reg}) appear to play a critical role in the progression of a number of cancers including breast cancer [13–17]. Even if tumor-specific effector cells are initially recruited into the tumor, the recruitment of T_{reg} cells into the same site can result in an unproductive anti-tumor response, and this is believed to play a major part in the failure of many cancer immunotherapies presently in development [14, 18]. Furthermore, recent research in mouse and human showed that, regardless of the overall number of T_{reg} cells in the tumor, the local balance of T_{reg} to effector T cells (T_{eff}) is critical to the success of immunotherapy and ultimately survival [19–22]. The murine CT26 colon carcinoma cell line has been shown to be a potent inducer of immunosuppressive mechanisms [23–25] making it a good model to evaluate the interplay between anti-tumor immunity and tumor-induced immunosuppression. When injected intravenously (i.v.), CT26 cells give rise to experimental pulmonary metastasis while mediating the infiltration of tumor-bearing lungs by a large number of IL-10-secreting T_{reg} [25]. Data described here demonstrate that MVA-BN[®]-HER2 treatment induced Th1-dominated HER-2-specific cellular and humoral immunity, reduced the amount of T_{reg} cells in the tumor and altered the intratumoral balance of effector and regulatory T cells resulting in potent anti-tumor efficacy against CT26-HER-2 tumors. Our findings suggest that MVA-BN[®]-HER2 generated an anti-tumor response in line with the current paradigm of an “immune signature” [26] that could lead to protective immunity in humans and warrants further testing of MVA-BN[®]-HER2 in clinical trials.

Materials and methods

Animals, tumor cell lines, MVA-BN[®]-vectors and HER2 proteins

Female BALB/c (H-2^d) mice were obtained from Harlan Sprague–Dawley, Inc. CT26-HER-2 is a cell line derived from an undifferentiated murine colon adenocarcinoma that was transduced with human HER-2/neu [27]. CT26-HER-2 cells were grown in IMDM + 10% bovine calf serum (Cellgro, Mediatech, VA). MVA-BN[®] and MVA-BN[®]-HER2 were produced for BNIT by Bavarian Nordic (BN; Martinsried, Germany) [28]. The HER2 transgene encodes the extracellular domains of HER-2 and a portion of the trans-membrane domain but lacks the intracellular domains. Furthermore, it encodes the T helper cell epitopes p2 and p30 from tetanus toxin [2]. The HER2 protein used in combination with CFA is similar to the protein expressed by the MVA-BN[®]-transgene with the exception that the trans-membrane domain (9 AA) is absent and the protein has a C-terminal His tag.

Analysis of immune responses

Serum antibody titers and the frequency of IFN- γ -producing T cells were determined by standard ELISA and ELISPOT, respectively. Functionality of the T cells was tested by *in vivo* CTL assay and intracellular IFN- γ stain (for details, see Online Resource “ESM_1_Materials and Methods” MOESM1: Supplemental material 1).

Immunotherapy

SE5 CT26-HER-2 cells were injected i.v. into female BALB/c mice. On day four, mice were treated intraperitoneally (i.p.) with 10 μ g HER2 in CFA or SE7 TCID₅₀ of MVA-BN[®] or MVA-BN[®]-HER2 unless indicated otherwise in the Figure legend. Fourteen days after tumor implantation, mice were euthanized, bled by cardiac puncture, and lungs and spleens were collected. For assessment of tumor burden, lungs were washed in PBS, tapped dry on a tissue and weighed. The tumor burden was calculated by subtracting the average lung weight of naïve mice from the average lung weight of tumor-challenged TBS-treated mice [29]. % tumor reduction was calculated as: ((average lung weight of treated group – naïve group)/(average lung weight of TBS-treated group – naïve group)) \times 100.

In vivo depletions

Mice were injected i.p. with 100 μ g of anti-CD4 (GK1.5), anti-CD8 (2.43), anti-CD25 (PC61) or isotype control

Fig. 1 Therapeutic anti-tumor efficacy of MVA-BN[®]-HER2, MVA-BN[®]-HER2 or HER2+CFA. **a** Representative pictures (a–c) and H&E-stained sections (40×, d–f) of lungs from naïve animals (no tumor challenge; top row) or tumor-challenged mice treated with TBS (middle row) or 1E7 TCID₅₀ MVA-BN[®]-HER2 (bottom row). **b** Lung weights. Average lung weights (±SD) of each group and the % tumor reduction are indicated at the bottom of the graph (black bars demonstrate the median lung weight of each group). Mice were treated on day 4 with 1E7 TCID₅₀ MVA-BN[®]-HER2, MVA-BN[®] or 10 µg HER2 protein formulated in CFA. Similar results were achieved in several repeat studies. **c** Survival study: Mice (*n* = 10/group) were challenged with 5E3 CT26-HER-2 cells on day 1 and treated q1 week × 4 with MVA-BN[®]-HER2 (1E7 TCID₅₀) starting on day 4 (d4, 11, 18, 25) (D4) or on day 8 (d8, 15, 21, 28) (D8). TBS treatment was started on day 4

antibody starting 3 days before tumor implantation and then every 2–3 days throughout the study. Alternatively, 250 µg of antibody was given weekly. Depletion was confirmed on days 1 and 8 (on spleens and lungs from 2 mice per group) and at the end of study when FACS analysis was performed.

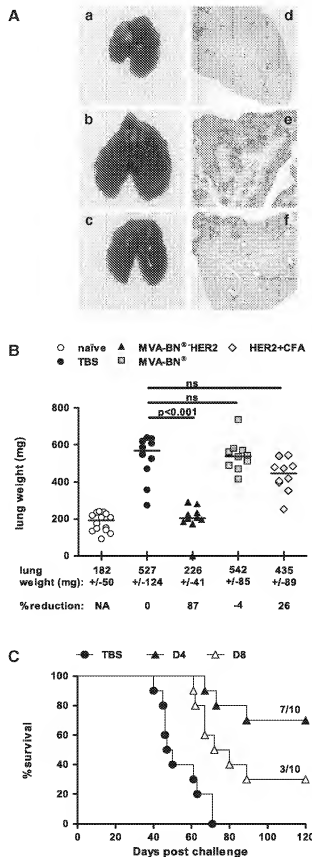
Statistical analysis

Statistical significance between treatment groups was evaluated by One-Way ANOVA followed by Bonferroni's Multiple Comparisons post-test. Log-rank test was performed for survival curves.

Results

Treatment with MVA-BN[®]-HER2 induces potent therapeutic efficacy against established CT26-HER-2 tumors

The immunotherapeutic efficacy of MVA-BN[®]-HER2 was evaluated in the CT26-HER-2 pulmonary metastasis model. Mice were challenged i.v. with 5E3 CT26-HER-2 cells and treated 3 days later with a single dose of either 1E7 TCID₅₀ of the parental MVA-BN[®] vector, MVA-BN[®]-HER2 or 10 µg of HER2 protein formulated in Complete Freund's Adjuvant (HER2+CFA). Control animals received TBS. Tumor burden of all mice was determined on day 15. As shown in Fig. 1a, lungs of TBS-treated mice were dramatically enlarged by the growing tumor and individual foci had already fused and were aggressively growing throughout the lung tissue (panels b and e). Strikingly, the lungs of animals treated with a single dose of MVA-BN[®]-HER2 had very small or no visible tumors by gross examination, and only a few small foci of tumor cells were visible by histology (panels c and f). While the average lung weight of tumor-challenged TBS-treated mice was almost threefold higher than that of naïve



lungs, MVA-BN[®]-HER2 treatment had potent anti-tumor activity and inhibited tumor growth by 87% ($P < 0.001$, Fig. 1b). In contrast, tumor burden was not statistically different between mice of the TBS- and MVA-BN[®]-treated groups, demonstrating that anti-tumor efficacy was dependent on antigen-specific responses. Treatment with HER2+CFA showed non-statistically significant anti-tumor activity compared to no treatment (26% reduction in tumor burden). Hence, therapeutic intervention with MVA-BN[®]-HER2, but not with HER2+CFA ($P < 0.001$, Fig. 1b), resulted in the induction of protective HER-2-specific immune responses capable of suppressing aggressively growing HER-2-expressing tumors in mice. The anti-tumor activity of MVA-BN[®]-HER2 was further evaluated by monitoring mouse survival in two different model settings. In the first setting, mice were challenged with 5E5 CT26-HER-2 cells as described in Fig. 1b. Under these conditions, all control mice died by day 17 and the median survival of MVA-BN[®]-HER2-treated mice was approximately 25 days (see Fig. 3c). In the second and less aggressive setting, mice were challenged with lower tumor cell numbers (Fig. 1c). Under the latter conditions, 70% of mice treated with MVA-BN[®]-HER2 survived at least 120 days when treatment was started on day 4 ($P < 0.0001$). Furthermore, when the onset of treatment was delayed to day 8, significant protection was still observed with a survival rate of 30% 120 days after the tumor challenge ($P < 0.0011$).

MVA-BN[®]-HER2 induces potent Th1-dominated immune responses resulting in infiltration of tumor-bearing lungs by highly activated, HER-2-specific, CD8⁺CD11c⁺ T cells

The modalities by which MVA-BN[®]-HER2 triggers protective immunity were evaluated in immunogenicity studies. As shown in Supplemental Figures 1 and 2, both MVA-BN[®]-HER2 and HER2+CFA treatments induced anti-HER-2 antibody and T-cell responses. However, these responses differed qualitatively in that MVA-BN[®]-HER2 induced a strongly Th1-dominated humoral response with an IgG2a/

IgG1 ratio 1,000-fold higher than in HER2+CFA-immunized mice. Furthermore, treatment with MVA-BN[®]-HER2 induced five to tenfold higher frequencies of IFN- γ -producing, HER-2-specific T cells than HER2+CFA treatment. High amounts of TNF- α were also detected in supernatants of HER-2 restimulated splenocytes from MVA-BN[®]-HER2-treated animals, while HER2+CFA treatment induced the production of IL-5 (Supplemental Fig. 2e). Overall, these data are in line with the known Th1 adjuvant quality of the MVA-BN[®] vector.

To further identify the mechanism of MVA-BN[®]-HER2-mediated anti-tumor activity, lymphocytes infiltrating the lungs of tumor-challenged mice were characterized. The most striking result of treatment with MVA-BN[®]-HER2 or MVA-BN[®] was a strong accumulation of CD8 T cells. Indeed, 27.1 and 21.1% of lymphocytes were CD8⁺ in MVA-BN[®]-HER2- or MVA-BN[®]-treated animals, respectively. In contrast, CD8 T cells in lungs of TBS controls or lungs from HER2+CFA-treated animals were only marginally increased as compared to naïve lungs (Table 1).

While treatment with both viral vectors resulted in a high density of CD8 T cells in the lung, only MVA-BN[®]-HER2 markedly increased the number of activated effector/memory CD8 T cells as measured by high CD44 expression levels (40.4%, Table 1; Fig. 2a). In contrast, CD8 T cells from lungs treated with MVA-BN[®] showed similar levels of activation as naïve or HER2+CFA-treated mice. Notably, CD8 T-cell activation was reduced in tumor-bearing, TBS-treated animals, where only 7% of CD8 T cells were CD44^{high} (a 53% reduction compared to naïve animals), possibly due to immunosuppressive activities provoked by the growing tumor.

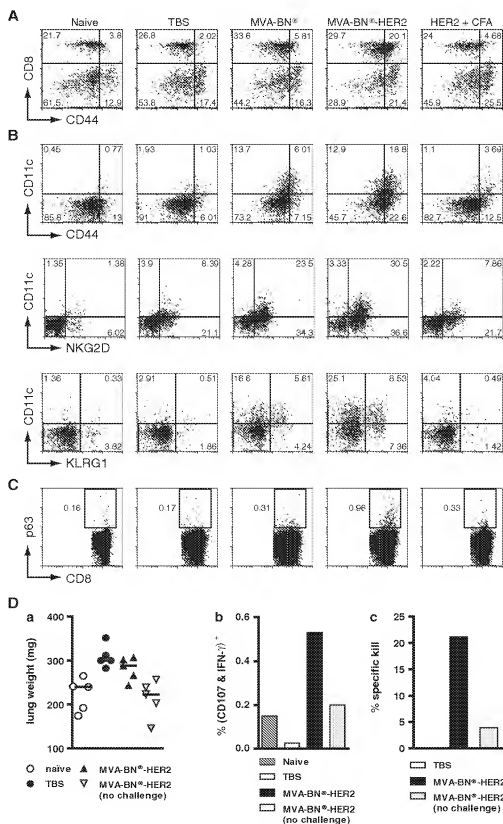
In contrast to CD8 T cells, the frequency of CD4 T cells remained relatively similar in all groups, although it was somewhat increased in lungs of HER2+CFA-treated animals (Table 1). However, the frequency of effector/memory CD4 T cells was highest in lungs of mice treated with MVA-BN[®]-HER2 (42.5%) followed by treatment with HER2+CFA (35.7%). MVA-BN[®] only marginally increased CD4 T-cell activation above levels seen induced by the growing tumor (26.9 vs. 24.4% in TBS-treated animals).

Table 1 Immunophenotyping of tumor-infiltrating T cells

Cell phenotype	Naïve	TBS	MVA-BN [®]	MVA-BN [®] -HER2	HER2+CFA
% CD8 ⁺ T cells of lymphocytes ¹	9.8	12.7	21.1	27.1	13.5
% CD4 ⁺ T cells of lymphocytes ¹	24.5	25.5	25.4	23.2	28.9
% CD44 ^{high} of CD8 ⁺ T cells ²	14.9	7	14.7	40.4	16.3
% CD44 ^{high} of CD4 ⁺ cells ²	17.3	24.4	26.9	42.5	35.7
% NKG2D ⁺ of CD8 ⁺ CD11c ⁺ T cells ³	50.0	68.0	84.5	90.2	77.9

¹ Percent cells have been calculated as the % of CD3⁺CD4⁺ or CD3⁺CD8⁺ lymphocytes. ² Calculated as % CD44^{high} of all CD4⁺ or CD8⁺ cells seen in Fig. 2 panel A. ³ Calculated as % NKG2D⁺ of CD8⁺CD11c⁺ T cells (Fig. 2). Similar results were found in 4 independent studies

Fig. 2 Immunophenotyping of lung-infiltrating CD8⁺ T cells. FACS analysis of CD8⁺ T cells from lungs of mice treated as described in Fig. 1b. Similar results were achieved in 4 independent experiments using either 1E7 or 5E7 TCID₅₀ of either virus. Gates **a**: CD3⁺ lymphocytes; **b** and **c**: CD3⁺CD8⁺ lymphocytes. **d** Functionality of p63-specific CD8 T cells isolated from lungs of MVA-BN[®]-HER2-treated animals. **Panel a** Lung weight in mg at day 11 after tumor challenge. **Panel b** Intracellular IFN- γ stain after 24 h *in vitro* restimulation with p63. **Panel c** p63-specific *in vivo* CTL activity. Lungs of 5 mice were pooled for both assays



Additional phenotyping of T cells infiltrating the lungs of MVA-BN[®]-HER2-treated animals revealed a high proportion of CD8⁺CD11c⁺ effector/memory cells. As shown in Fig. 2b, 18.8% of CD3⁺CD8⁺ T cells were CD11c⁺CD44^{high}. This constitutes an approximately 20-fold increase in this specific cell population compared to naïve lungs or TBS-treated tumor-bearing animals. CD11c⁺CD44^{high} cells were also detectable in the lungs of MVA-BN[®] or HER2+CFA-treated animals; however, the frequency of these cells was much lower (6.01 and 3.69%, respectively). Furthermore, the CD8⁺CD11c⁺-positive T cells found in the lung expressed NKG2D and KLRG-1, two markers often found on activated effector cells. As shown in panel 2 of Fig. 2b, the majority of CD8⁺CD11c⁺ T cells expressed NKG2D in each of the tumor-bearing groups, with a maximal percentage of 90% expressing NKG2D in the lungs of MVA-BN[®]-HER2-treated animals (Table 1). Interestingly, the expression of KLRG1 (a marker of CD8⁺ effector T cells commonly induced in viral infections [30]) was specific for CD8⁺CD11c⁺ T cells isolated from the lungs of MVA-BN[®] or MVA-BN[®]-HER2-treated animals.

To measure HER-2-specific T-cell responses, lymphocytes isolated from lungs or spleens were analyzed using a pentamer specific for the mouse MHC class I molecule K^b loaded with the immunodominant HER-2 peptide p63. As shown in Fig. 2c, the growing tumor alone did not induce significant amounts of p63-specific CD8⁺ T cells (TBS 0.17 vs. 0.16% in naïve lungs). In contrast, the percentage of p63-specific CD8⁺ T cells in the lungs of MVA-BN[®]-HER2-treated animals rose markedly to approximately 1%. Similar to the whole population of CD8⁺ T cells from the lungs of MVA-BN[®]-HER2-treated animals, 33% of p63-specific CD8⁺ T cells were CD11c⁺ and 43% were CD44^{high} (data not shown). In comparison, only a slight elevation in p63-specific CD8⁺ T cells was observed in the lungs of MVA-BN[®] (0.31%) or HER2+CFA-treated mice (0.33%). Importantly, HER-2-specific CD8⁺ T cells appeared to accumulate specifically in tumor-bearing lungs since p63 staining was not detected above background in the spleens of any treatment group (data not shown). The functionality of the p63-specific CD8⁺ T cells in the lungs of MVA-BN[®]-HER2-treated mice was tested during the effector phase of the response (6–7 days post-treatment). As shown in Fig. 2d (panel a), the tumor burden was similar between TBS- and MVA-BN[®]-HER2-treated groups at this time point (day 11). Restimulation with the p63 peptide resulted in the production of IFN- γ and CD107 by CD8⁺ T cells only in cells from the lungs of MVA-BN[®]-HER2-treated animals (Fig. 2d panel b). Furthermore, p63-labeled target cells were killed in vivo by tumor-challenged MVA-BN[®]-HER2-treated animals (Fig. 2d panel c). Notably, when non-challenged mice were treated with MVA-BN[®]-HER2, p63-specific T cells could only be found in the spleens (data not shown) but not in the lungs of this group of mice, demonstrating that the tumor is required for the homing

of p63-specific T cells to the tumor. Taken together, these data demonstrate that MVA-BN[®]-HER2 treatment led to tumor infiltration by highly activated and functional (CD44^{high}, NKG2D⁺, KLRG1⁺), HER-2-specific, CD8⁺CD11c⁺ T cells, while HER2+CFA treatment preferentially activated CD4⁺ T cells.

MVA-BN[®]-HER2 anti-tumor activity overrides the tumor-mediated accumulation of T_{reg} cells in the lung

The reduced percentage of CD8⁺ T-cell activation in TBS-treated animals suggested that the growing tumor exerted immunosuppressive activities. In line with this, FoxP3 staining of lung-infiltrating CD4⁺ T cells revealed that the growth of CT26-HER-2 tumors in the lung was accompanied by a significant increase in the percentage of T_{reg} cells from 2.22% (or 10.8% of CD4⁺ T cells) in naïve lungs to 5.79% (or 26.1% of CD4⁺ T cells) in tumor-bearing, TBS-treated mice (Table 2, line 1 and 4). Treatment of tumor-challenged mice with MVA-BN[®]-HER2 reduced the frequency of T_{reg} cells to 3.44%. In contrast, treatment of tumor-bearing animals with MVA-BN[®] affected the frequency of T_{reg} cells only marginally (4.89 vs. 5.79%), while HER2+CFA treatment slightly increased the frequency of T_{reg} cells to 6.47% of lymphocytes.

To assess how treatment affected the balance between T_{eff} cells and T_{reg} cells in the lung, the CD4⁺ and CD8⁺ effector T cell to T_{reg} cell ratios were calculated (Table 2). MVA-BN[®]-HER2 treatment significantly improved the ratio of both CD4⁺T_{eff}/T_{reg} cells and CD8⁺T_{eff}/T_{reg} cells. Furthermore, the ratio of activated CD8⁺CD44^{high}/T_{reg} cells showed an even more dramatic 30-fold increase in the MVA-BN[®]-HER2-treated group as compared to the TBS-treated group and a fivefold increase as compared to naïve lungs. Treatment with the parental vector MVA-BN[®] more modestly increased the ratio of CD8⁺T_{eff}/T_{reg} cells and CD8⁺CD44^{high}/T_{reg} cells as compared to TBS-treated mice but had little effect on CD4⁺T_{eff}/T_{reg} cell ratios. Treatment with HER2+CFA increased CD4⁺T_{eff}/T_{reg} ratios to a similar extent as MVA-BN[®] treatment but had only minor effects on CD8⁺T_{eff}/T_{reg} ratios. These data demonstrate that treatment with MVA-BN[®]-HER2, but not with HER2+CFA, reduced the frequency of tumor-induced T_{reg} cells in the lung and more importantly, led to a much higher ratio of effector to T_{reg} cells, particularly with respect to CD8⁺ T cells.

In vivo depletion confirms that CD8⁺ cells are required for the anti-tumor activity of MVA-BN[®]-HER2 and identifies T_{reg} cells as an immunosuppressive mechanism induced by the tumor

To further substantiate the role of CD8⁺ effector and T_{reg} cells in this tumor model, mice were depleted of CD4⁺ or

Table 2 Immunophenotyping of effector and regulatory T cells in the lung

	Naive	TBS	MVA-BN [®]	MVA-BN [®] -HER2	HER2+CFA
% CD4 ⁺ FoxP3 ⁺ (T _{reg}) of lymphocytes	2.22	5.79	4.89	3.44	6.47
% CD4 ⁺ FoxP3 ⁺ (T _{eff})	18.4	16.4	15.9	18.3	20.7
% CD4 ⁺ T cells of lymphocytes	20.62	22.19	20.79	21.74	27.17
% T _{reg} of CD4 ⁺ T cells	10.8	26.1	23.5	15.8	23.8
% reduction in tumor-induced T _{reg} (total lymphocytes) ¹			25.2	65.8	−19.04
% reduction in tumor-induced T _{reg} (of CD4 ⁺ T cells) ²			16.99	67.32	15
CD4 ⁺ T _{eff} /T _{reg} ratio	8.3	2.8	3.3	5.3	3.2
CD4 ⁺ CD44 ^{high} /T _{reg} ratio	1.9	1	1.4	3	1.5
% CD8 ⁺ T cells (T _{eff})	8	10.4	16.9	24	11.6
CD8 ⁺ T _{eff} /T _{reg} ratio	3.6	1.8	3.5	7	1.8
CD8 ⁺ CD44 ^{high} /T _{reg} ratio	0.6	0.1	0.6	3.1	0.3

All percentages are expressed as % of lymphocytes except for % T_{reg} of CD4⁺ T cells. Effector T cell (T_{eff}) to regulatory T cell (T_{reg}) cells ratios were calculated from FACS data: ((% CD4⁺Foxp3⁺ lymphocytes) or (% CD8⁺ lymphocytes)/(% CD4⁺Foxp3⁺)). Similar results were found in four independent studies. % reduction in tumor-induced T_{reg} cells was calculated from the (% CD4⁺FoxP3⁺ (T_{reg}) of lymphocytes)¹ or (% T_{reg} of CD4⁺ T cells)² as follows: tumor-induced T_{reg} = TBS – naive. % reduction = 100 – ((treatment group – naive)/tumor-induced T_{reg}) × 100

CD8⁺ cells beginning just prior to tumor challenge and throughout the entire course of the model. CD8 depletion reduced the anti-tumor activity of MVA-BN[®]-HER2 treatment from 99.6 to 42.4% (Fig. 3a). This difference was highly significant ($P < 0.001$) demonstrating the importance of CD8⁺ effector T cells in MVA-BN[®]-HER2-mediated anti-tumor efficacy. However, the anti-tumor activity of MVA-BN[®]-HER2 treatment was not completely abolished by CD8 depletion, as the mean lung weight from this group was still significantly smaller than those of CD8-depleted, TBS-treated mice ($P < 0.05$). This suggests that additional CD8-independent effector mechanisms contributed to the anti-tumor efficacy.

While CD8 depletion by itself had no effect on tumor growth (Fig. 3a), removal of CD4⁺ cells consistently resulted in a significant reduction in tumor burden even without MVA-BN[®]-HER2 treatment (Fig. 3a, b). Of note, combining CD4 depletion with MVA-BN[®]-HER2 treatment reliably decreased the anti-tumor activity of MVA-BN[®]-HER2 by 20–30% (Fig. 3a, b).

The decrease in tumor burden resulting from depletion of CD4⁺ cells in the absence of treatment implied that the increased percentage of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells present in the tumors (row 1, Table 2) play a key role in suppressing immune responses to the tumor. To test this directly, CD25⁺ cells were depleted by administration of an anti-CD25 mAb. CD25 depletion reduced tumor burden to a similar extent as CD4 depletion (35 and 48%, respectively, $P > 0.05$, Fig. 3b). This finding confirmed that the depletion of T_{reg} cells led to the reduction in tumor burden in CD4-depleted mice and substantiates the significance of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in this tumor model. However, the data also demonstrate that depletion

of T_{reg} cells alone does not result in effective tumor rejection.

The observed effects of CD4 or CD25 depletion on the therapeutic efficacy of MVA-BN[®]-HER2 were further distinguished by evaluating their effect on the survival rates of tumor-challenged mice. Mice were challenged with tumors and treated with MVA-BN[®]-HER2 weekly four times in the presence or absence of CD4 or CD25 depletion. All mice in the TBS group died between days 16 and 18. Treatment with MVA-BN[®]-HER2 significantly prolonged the median survival time from 17 to 24 days with one out of 15 mice surviving through day 60 when the study was terminated (Fig. 3c). CD4 depletion significantly reduced the median survival time from 24 to 21 days ($P = 0.0081$, Fig. 3c) supporting a role of CD4 effector cells in MVA-BN[®]-HER2-mediated anti-tumor immunity. In contrast, the depletion of CD25⁺ T cells prolonged the median survival time from 24 to 50 days resulting in significantly better survival than treatment with MVA-BN[®]-HER2 alone ($P = 0.0011$), presumably due to the more complete elimination of T_{reg} cells.

Induction of HER-2-specific CD8 T cells requires treatment with MVA-BN[®]-HER2 even in the absence of T_{reg} cells

Phenotypic analysis revealed a treatment-independent increase in overall CD8 T-cell activation in lungs of CD4⁺-depleted animals. Upon CD4 depletion, the percentage of activated CD8⁺ T cell rose from 5.6 to 26.7% in TBS-treated animals and from 12.8 to 29.6% in MVA-BN[®]-HER2-treated animals (Fig. 4a). The effect of CD4 depletion on the amplification of HER-2-specific CD8⁺ T

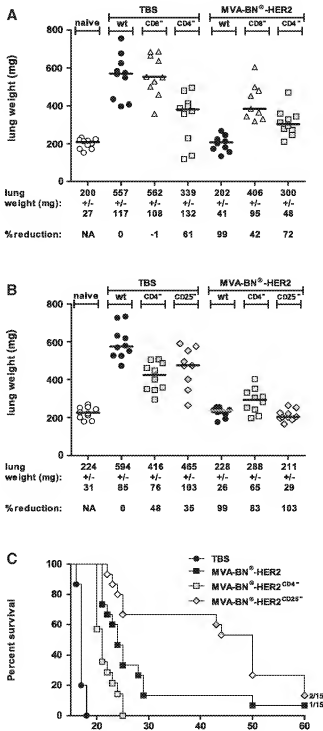
Fig. 3 Effect of in vivo depletion of CD8⁺, CD4⁺ or CD25⁺ T cells ▶ on anti-tumor efficacy. **a** Tumor challenge and treatments were performed as described for Fig. 1. A single dose SE7 TCID₅₀ MVA-BN[®]-HER2 was given on day 4. CD4 or CD8 T cells were depleted by i.p. injection of anti-CD4, anti-CD8, anti-CD25 or isotype control antibody. Treatment with isotype control Ab had no effect on tumor growth (data not shown). CD4 and CD8 T-cell depletion was confirmed by FACS analysis (days 1, 8 and 15). Depletion was 100% complete throughout the study. Similar results were achieved in three independent studies. **b** Separate experiment evaluating the role of CD25⁺ T cells. Similar results were achieved in two independent studies. **c** Survival study: Mice ($n = 15$ /group) were challenged with tumors on day 1 as described before and treated q1 week \times 4 (days 4, 11, 18 and 25) with TBS or MVA-BN[®]-HER2 (IE7 TCID₅₀) combined with CD4 or CD25 depletion. Antibodies were given on days -4, 3, 10, 17 and 24 at 250 μ g/dose. Statistical analysis was performed by log-rank test

cells, however, was dependent on treatment with MVA-BN[®]-HER2. No increase in HER-2-specific CD8⁺ T cells was observed in the context of CD4 depletion in TBS-treated mice (Fig. 4b). In contrast, the percentage of p63-specific CD8⁺ T cells reliably increased by two to threefold in lungs of CD4-depleted animals treated with MVA-BN[®]-HER2 (Fig. 4b).

In contrast to the depletion of all CD4⁺ cells, the functional depletion of T_{reg} cells by treatment with anti-CD25 antibody [31] showed only minor effects on the activation of CD8⁺ T cells in TBS-treated animals (Fig. 4a). A modest increase in the frequency of activated CD8⁺ T cells from 12.8 to 19% in the lung and 14.4–31.2% in the spleen was observed in MVA-BN[®]-HER2-treated animals. Notably, the increase in p63-specific CD8⁺ T cells was more pronounced in MVA-BN[®]-HER2-treated animals combined with CD25 depletion than in combination with CD4 depletion (Fig. 4b), again suggesting a supportive role of CD4 T cells in MVA-BN[®]-HER2-mediated anti-tumor efficacy.

Discussion

Successful cancer immunotherapy has to accomplish two goals: first, the induction of an immune response which is capable of killing cancerous cells; second, to combat or reverse the inflammatory/immunosuppressive mechanisms exerted by the growing tumor that lead to subsequent down-regulation of tumor-induced responses. Pertaining to this challenge, the anti-tumor activity of MVA-BN[®]-HER2 was evaluated in a pre-clinical tumor model known to create a strong immunosuppressive environment able to restrain T-cell responses to both tumor-specific and bystander antigens [23–25]. Notably, a single treatment with MVA-BN[®]-HER2 showed potent anti-tumor activity against CT26-HER-2 lung metastasis despite the unfavorable immunosuppressive environment. The comparison of HER-2-specific humoral and cellular immune responses



induced by treatment of mice with MVA-BN[®]-HER2 or HER2+CFA demonstrated that the MVA-BN[®] vector exerted potent Th-1 adjuvant activities. More importantly, the quality of the immune response is a crucial factor in cancer immunotherapy as only treatment with MVA-BN[®]-HER2 resulted in protective anti-tumor immunity in this aggressive tumor model, while treatment with HER2+CFA

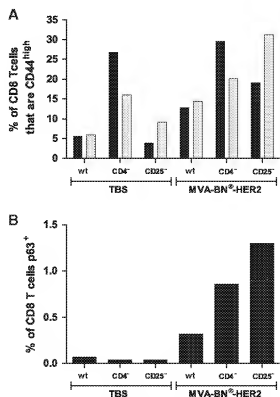


Fig. 4 Effect of CD4 or CD25 depletion on CD8 T-cell activation and frequency of HER-2-specific CD8 T cells. Animals were treated as described in legend to Fig. 3a, b. On day 15, single-cell suspensions were prepared from lungs and spleens for FACS analysis. **a** Percent of activated CD8⁺ T cells in lung (black bars) and spleen (gray bars). **b** Percent K⁺ p63 pentamer⁺ cells of CD8⁺ T cells; similar results were achieved in three independent studies

did not. Anti-tumor activity was accompanied by a high density of activated CD8⁺ T cells along with a clear reduction in the percentage of T_{reg} cells in the lungs of animals treated with MVA-BN[®]-HER2, thus resulting in a crucial shift in the balance of T_{eff} to T_{reg} cells in favor of effector T cells [22]. Importantly, the tumor-infiltrating HER-2-specific CD8 T_{eff} cells were functional as demonstrated by *in vivo* CTL activity and intracellular IFN- γ production. These cells were found in the spleen in MVA-BN[®]-HER2-treated non-challenged animals and homed to the lungs in tumor-challenged mice. Immunotherapy with MVA-BN[®]-HER2 also showed a significant anti-tumor efficacy in a solid CT26-HER2 tumor model (see Supplemental Fig. 3). In this model, epitope spreading to other tumor-associated antigens and long-lasting CTL memory was demonstrated.

A high percentage of CD8 T cells found in lungs of MVA-BN[®]-HER2- or MVA-BN[®]-treated mice expressed the markers CD11c, NKG2D and KLRG1. The expression of the myeloid marker CD11c on CD8 T cells has been described following acute infections with several viruses including vaccinia [32]. In general, all CD8⁺CD11c⁺ T

cells also expressed elevated levels of CD44 and have been described in virally infected mice as activated, virus-specific, cytotoxic T cells that are much more effective at controlling viral infections than CD8⁺ T cells negative for CD11c [32–34]. Interestingly, CD8⁺CD11c⁺ T cells have also been identified as effectors of anti-4-1BB-mediated tumor suppression in several mouse tumor immunotherapy models using agonistic anti-4-1BB antibodies [35–37]. Similar to the results described here, both NKG2D and KLRG-1 were highly expressed on anti-4-1BB-induced CD8⁺CD11c⁺ T cells, and they preferentially accumulated in the tumor tissue. Together, these data suggest that CD8⁺CD11c⁺ T cells found in tumor-bearing lungs are a mixture of highly activated MVA virus- or tumor-specific CD8⁺ effector/memory T cells.

The lung in particular is an immunosuppressive environment in which the constitutive production of IL-10 is necessary to protect the integrity of the delicate and highly vascularized tissue. Growing CT26 tumors enhance this already immunosuppressive environment resulting in the recruitment of CD4⁺CD25⁺FoxP3⁺T_{reg} cells [25]. Natural CD4⁺CD25⁺T_{reg} play a critical role in the progression of a number of cancers [17] and are believed to play a major part in the failure of many immunotherapies against tumors in mice [24, 38–40] and men [18]. We found that the presence of CT26-HER2 tumors in the lung consistently increased the percentage of T_{reg} cells by approximately threefold. The fact that a single treatment with MVA-BN[®]-HER2 significantly reduced the frequency of T_{reg} cells by up to 70% demonstrates that treatment with MVA-BN[®]-HER2 can override the potent effects of CD4⁺CD25⁺FoxP3⁺T_{reg} cells in establishing conditions conducive to tumor growth, even in the immunosuppressive environment of the lung. In stark contrast, treatment with HER2-CFA resulted in a further increase in the percentage of T_{reg} cells in the lung and failed to induce any significant anti-tumor efficacy in this model. *In vivo* depletion experiments further confirmed that the importance of T_{reg} cells in this tumor model as depletion of either CD4⁺ T cells or CD25⁺ T cells resulted in a similar partial reduction in tumor burden. MVA-BN[®]-HER2 therapy was necessary for the expansion of p63-specific CD8 T cells in both CD4- and CD25-depleted animals, demonstrating that the tumor alone was not able to induce HER-2-specific T cells even in the absence of T_{reg} cells. However, phenotypic analysis of lung-infiltrating T cells from mice depleted of either CD25⁺ or CD4⁺ T cells revealed interesting differences. While CD4 depletion resulted in an increase in CD8 T-cell activation irrespective of treatment with MVA-BN[®]-HER2, the depletion of CD25⁺ cells only increased CD8 T-cell activation in MVA-BN[®]-HER2-treated mice. Although the increased activation of CD8 T cells could be responsible for the reduced tumor burden

observed in CD4-depleted mice, it is unlikely that CD8⁺ T cells are responsible for the reduced tumor size observed in TBS-treated CD25-depleted mice. This could suggest that the absence of T_{reg} cells might relieve immunosuppressive mechanisms that allow for the activation of cell types other than CD8 T cells. Two likely candidates are CD4 T cells or innate immune effector cells such as NK cells. We are currently investigating the role of CD4 T cells and innate immune mechanisms in MVA-BN[®]-HER2-mediated anti-tumor efficacy.

In cancer immunotherapy, a broad response involving all arms of the immune system is desired. Repeated treatment of mice with MVA-BN[®]-HER2 resulted in the induction of robust anti-HER-2 antibody responses (supplemental data Fig. 1). Using trastuzumab as a standard, we determined that these titers correlate with 50–500 µg HER-2-specific antibody/mL serum (data not shown), a level within the range of that found in patients treated with trastuzumab [41, 42]. Although HER-2-specific antibodies do not seem to play a protective role in this particular mouse tumor model, they can be important in other models (our own unpublished observations) and a role for antibody-mediated rejection of HER-2-positive tumors has been well established by the therapeutic activity of trastuzumab. Several other monoclonal antibodies have also demonstrated clinical effectiveness in a variety of malignancies through mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [43, 44]. Antibody response induced by MVA-BN[®]-HER2 showed a strong Th1 bias with IgG2a to IgG1 ratios 500- to 1,000-fold higher than in mice immunized with HER2+CFA. Since Th1 isotypes are thought to be the most efficient mediators of ADCC and CDC, the humoral responses produced by MVA-BN[®]-HER2 treatment are desirable and may provide therapeutic activity in human patients.

In conclusion, the data presented here demonstrate that a single treatment with MVA-BN[®]-HER2 was able to simultaneously induce Th1-dominated HER-2-specific immune responses and control tumor-induced immunosuppression resulting in potent anti-tumor efficacy. These preclinical results and the excellent safety profile and immunogenicity of MVA-BN[®] even in immune-compromised patients (4–6) provide further support for the development of MVA-BN[®]-HER2 for cancer immunotherapy. Notably, the immune responses induced by immunotherapy with MVA-BN[®]-HER2 are in line with a recently described distinct “immune signature” necessary for the inhibition of a variety of solid tumors treated with different immunotherapeutic strategies including breast cancer [26, 45–48]. Two hallmark features of this signature are the generation of a tumor-specific Th1 responses and a high density of T cells in the tumor parenchyma. The

ability of MVA-BN[®]-HER2 to induce HER-2-specific immune responses in humans is currently being assessed in Phase I/II clinical trials in HER-2-positive breast cancer patients (NCI00485277).

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Conflict of interest The authors declare that they are employees and stock holders of BN ImmunoTherapeutics.

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